

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE (DD-MM-YYYY) 2004		2. REPORT TYPE Open Literature		3. DATES COVERED (From - To)	
4. TITLE AND SUBTITLE Cross-linking Interferes with Assessing Sulfur Mustard-induced DNA Damage in Human Peripheral Blood Lymphocytes using the Comet Assay				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Moser, J, Levine, CE, Thomas-Dunmeyer, DR, Smith, WJ				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) US Army Medical Research Institute of Chemical Defense ATTN: MCMR-UV-DB 3100 Ricketts Point Road Aberdeen Proving Ground, MD 21010-5400				8. PERFORMING ORGANIZATION REPORT NUMBER USAMRICD-P00-004	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) US Army Medical Research Institute of Chemical Defense ATTN: MCMR-UV-RC 3100 Ricketts Point Road Aberdeen Proving Ground, MD 21010-5400				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES Published in Toxicology Mechanisms and Methods, 14, 195-202, 2004. Author's current address is USAMMDA, ATTN: MCMR-UMP, 622 Neiman Street, Fort Detrick, MD 21702-5009.					
14. ABSTRACT See reprint.					
15. SUBJECT TERMS Comet assay, DNA cross-linking, DNA damage, lymphocytes, sulfur mustard					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UNLIMITED	18. NUMBER OF PAGES 8	19a. NAME OF RESPONSIBLE PERSON LTC Janet Moser
a. REPORT UNCLASSIFIED	b. ABSTRACT UNCLASSIFIED	c. THIS PAGE UNCLASSIFIED			19b. TELEPHONE NUMBER (include area code) 301-619-8402

Cross-Linking Interferes with Assessing Sulfur Mustard-Induced DNA Damage in Human Peripheral Blood Lymphocytes Using the Comet Assay

Janet Moser, Claire F. Levine, Delvena R. Thomas-Dunmeyer,
and William J. Smith

Pharmacology Division, U.S. Army Medical Research Institute of Chemical Defense,
3100 Ricketts Point Road, Aberdeen Proving Ground, Maryland 21010-5400

Sulfur mustard (SM) is a blistering agent that produces DNA strand breaks. To detect SM-induced DNA single strand breaks in human peripheral blood lymphocytes (PBL), cells were exposed to various concentrations of SM (10 to 1000 μM), and the comet assay (single-cell gel electrophoresis) was performed. We observed a SM concentration- and time-dependent increase in detectable DNA damage. To test whether SM-induced DNA cross-linking inhibits DNA migration in the comet assay, PBL were exposed to a) SM alone (10 to 1000 μM), b) H_2O_2 (0.001%), which produces DNA single strand breaks with no cross-links, or c) SM followed at 2, 4, or 6 h by H_2O_2 . With H_2O_2 alone, a large amount of strand breakage was detected. With H_2O_2 plus SM, detectable H_2O_2 -induced strand breaks decreased as SM concentration increased up to 30 μM ; at 30 μM and above, the response with H_2O_2 plus SM was similar to that with SM alone. Interference with the detection of H_2O_2 -induced DNA strand breaks appears to be SM concentration-dependent up to 30 μM , and independent of SM concentration at $\geq 30 \mu\text{M}$. This is presumably due to SM-induced cross-linking. It follows that cross-linking in DNA of SM-exposed PBL also interferes with DNA migration and detection of DNA strand breaks when cells are exposed to SM alone.

Keywords Comet Assay, DNA Cross-Linking, DNA Damage, Lymphocytes, Sulfur Mustard

Sulfur mustard (SM) is a cytotoxic vesicant chemical warfare agent, or “blister agent,” that produces severe lesions on exposed epithelial tissues. There is, to date, no effective antidote for SM. The most sensitive target of SM is cellular DNA. SM preferentially alkylates at the N-7 position of guanine or the

N-3 position of adenine, and alkylation leads to depurination of DNA strands. Subsequent breakage of phosphodiester bonds at the apurinic sites produces DNA single-strand breaks (reviewed in Papirmeister et al. 1991). To repair DNA breaks, the resulting gap in the DNA chain is filled with appropriate bases (DNA polymerization), and these added bases are then ligated to complete the DNA strand repair.

Interstrand or intrastrand DNA cross-links can form when a guanine-alkyl-guanine adduct is produced by the alkylation of 2 guanines on opposite strands or 2 guanines on the same strand, respectively (Reid and Walker 1969; Walker 1971). In DNA isolated from SM-exposed cells, approximately 75% of these cross-links are intrastrand (Walker 1971). The excision rate of SM-induced DNA cross-links is logarithmic with a half-life of approximately 2 h; a rapid exponential reduction in cross-links occurs between 1 and 6 h after SM exposure (Reid and Walker 1969; Walker and Reid 1971).

We used the comet assay, also known as single-cell gel electrophoresis, to detect DNA single-strand breaks in SM-exposed human peripheral blood lymphocytes (PBL). The comet assay is a fast, efficient, and very sensitive method for detecting DNA single-strand breaks in individual cells (Singh et al. 1988; de With et al. 1994; Slamenová et al. 1997). This assay can be used to monitor the rate of DNA repair by measuring the disappearance of DNA single-strand breaks over time (Fairbairn et al. 1995).

To perform the comet assay, nucleated cells are embedded in agarose gel on microscope slides and lysed by detergents at high salt concentrations. The cellular DNA is treated with a strong alkaline buffer to produce single-stranded DNA, electrophoresed under alkaline conditions, and stained with a fluorescent dye for visualization. Individual cells are analyzed with a fluorescence microscope and image analysis system. DNA of an undamaged cell appears as a spherical mass occupying the cavity formed by the lysed cell. When DNA is damaged, the negatively charged pieces of DNA are free to migrate in the electric field toward the anode and are seen streaming from the spherical mass (the

Received 5 December 2002; accepted 3 May 2003.

The authors thank Tony Callan, Naomi Campbell, Charlene Corun, Juanita Guzman, Eric Nealley, and Marian Nelson for technical assistance and Robyn Lee for assistance with statistical analysis.

Address correspondence to Dr. Janet Moser, U.S. Army Medical Materiel Development Activity, ATTN: MCMR-UMP, 622 Neiman Street, Fort Detrick, MD 21702-5009, USA. E-mail: janet.moser@us.army.mil

comet head) in the shape of a comet tail (Kent et al. 1995). Single-stranded DNA migrate according to molecular length (Freeman et al. 1986), with shorter DNA (that with more damage) migrating further from the nucleus than longer DNA (that with less damage). The comet moment (product of comet tail length and intensity of the tail) describes the relative amount of DNA strand breakage in a cell, and it has been correlated quantitatively with the frequency of DNA strand breaks (Olive and Banath 1993). The sensitivity of the comet assay is 0.1 break per 10^9 daltons, or 6.6 breaks per average chromosome, in UV-C damaged cells (Gedik et al. 1992).

Cross-linking agents can reduce or entirely inhibit detection of DNA damage in the comet assay by inhibiting DNA migration through the agarose gel. Known cross-linking agents such as nitrogen mustard (a compound closely related to sulfur mustard), cisplatin, and mitomycin C have been reported to reduce, in a concentration-dependent manner, DNA migration in cells containing DNA single-stranded breaks (Olive et al. 1992; Olive and Banath 1995; Pfuhler and Wolf 1996).

In this study, we found that the comet assay did not detect DNA damage in PBL at the low SM concentrations or the early time points following exposure as reported using alternative methods (2 h after exposure to 300 μ M SM or 24 h after exposure to 6 μ M SM; Meier and Millard 1998). We hypothesized that SM-induced DNA cross-linking inhibits DNA migration in the comet assay, resulting in decreased detection of DNA strand breaks. We tested our hypothesis by determining whether SM interferes with the migration of DNA from PBL exposed to H_2O_2 , an oxidizing agent that produces a random distribution of DNA single-strand breaks with no cross-links (Szmigiero and Studszian 1988).

MATERIALS AND METHODS

PBL Isolation

Human peripheral blood lymphocytes were acquired from normal adult volunteers under an approved human use protocol. PBL were isolated from whole blood (≤ 200 ml) by differential centrifugation on a Percoll gradient (Pharmacia Biotech, Piscataway, NJ, USA; density = 1.080 g/ml at 20°C) (Meier et al. 1987). Cells were washed twice with calcium-free and magnesium-free Tyrode's buffer and once with RPMI 1640 medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 0.05 mg gentamicin/ml. Between each wash, the cells were centrifuged at $900 \times g$ for 20 min at 20°C. The number of PBL obtained was determined by counting the cells on a hemocytometer. Following the final centrifugation, the isolated PBL were resuspended at 2×10^7 cells/ml in RPMI 1640 medium. The cells were then dispensed into 24-well tissue culture plates to yield a final concentration of $2-3 \times 10^6$ cells/well.

SM and H_2O_2 Treatment of PBL

Sulfur mustard with a purity of $\geq 98\%$ was obtained from the Edgewood Chemical and Biological Center (Aberdeen Proving

Ground, MD, USA). Stock solutions of SM were prepared as 4 mM solutions in RPMI 1640 medium.

PBL were exposed to seven SM concentrations ranging from 10 to 1000 μ M by diluting stock SM in RPMI 1640 medium and immediately adding aliquots to PBL cultures (Meier and Johnson 1992). The PBL were exposed to SM directly after plating into 24-well plates. Final volume was 1 ml/well.

To allow venting of volatile SM and permit its complete hydrolysis, control and exposed cells were incubated at room temperature (20 to 22°C) for 1 h after exposure in a fume hood. Following room temperature incubation, the lymphocytes were transferred to a 37°C, 5% CO_2 -95% air incubator until harvested.

PBL were exposed to a) SM alone, as described, b) H_2O_2 (0.001%) for 5 min at 2, 4, or 6 h after PBL isolation, or c) SM followed at 2, 4, or 6 h by a 5-min exposure to 0.001% H_2O_2 . Unexposed cells served as the negative control for DNA damage.

Harvesting PBL

PBL exposed to SM alone were harvested at 2, 4, or 6 h after exposure. PBL exposed to H_2O_2 or to SM plus H_2O_2 were harvested at 5 min after H_2O_2 exposure. After trituration several times, the PBL were suspended in RPMI 1640 medium and transferred from each well of the 24-well plate into individual 1.5-ml microcentrifuge tubes. The cells were centrifuged at $3500 \times g$ for 5 min at 4°C and resuspended in 600 μ l RPMI 1640 medium.

Viability Determination

After harvesting the cells, a 200- μ l aliquot from each PBL sample was dispensed into a 12 \times 75 mm test tube and diluted by the addition of 200 μ l of RPMI 1640 medium to each tube. Then 50 μ l of propidium iodide (Sigma Chemical Company, St. Louis, MO, USA; dissolved in RPMI 1640 medium at 0.3 mg/ml) was added to each tube containing PBL. After a 2- to 5-min room temperature incubation in the dark, propidium iodide incorporation into the cells was assessed using a FACSsort flow cytometer (Becton Dickinson, San Jose, CA, USA). The data were analyzed with the Lysis II computer software program (Becton Dickinson), and viability results were expressed as the percent of the total number of cells that excluded propidium iodide (Smith et al. 1991; Clayson et al. 1993).

Comet Assay

The comet assay was performed as described (Singh et al. 1988) with some modifications. Fully frosted microscope slides (Fisher Scientific, Pittsburgh, PA, USA) were covered with a base layer of 1% normal melting agarose (Type I; Sigma Chemical Company, St. Louis, MO, USA) in phosphate buffered saline (PBS; 0.15 M NaCl, 3 mM Na_2HPO_4 , both from Sigma; pH 7.4), and the slides were completely air dried at room temperature. A sample of the PBL suspension harvested from one well of the 24-well plate was diluted 1:10 in 1% low melting agarose

(Sea Plaque, FMC Bioproducts, Rockland, ME, USA) in PBS. Then 75 μ l of the cell-agarose mixture was added as a second layer onto the microscope slide and covered with a No. 1 coverslip (Corning, Inc., Horseheads, NY, USA; 24 \times 60 mm). The agarose was allowed to solidify on the slides at room temperature for approximately 15 min. After removing the coverslip, a third layer of agarose was formed on each slide by the addition of 100 μ l of 1% low melting agarose. The coverslip was immediately replaced, and the agarose was allowed to solidify at 4°C for at least 5 min.

To lyse the cells, the coverslips were removed, and the slides were submerged in cold (4°C) lysis solution (2.5 M NaCl, 100 mM EDTA, 1% N-lauroylsarcosine, 10 mM Tris base, pH 10, and 1% Triton X-100 added fresh, all from Sigma) and incubated at 4°C for a minimum of 1 h and a maximum of 3 days. The lysis solution was then poured from the slides. To denature the DNA, the slides were submerged in cold (4°C) electrophoresis solution (300 mM NaOH, 1 mM EDTA, both from Sigma; pH \geq 13) and incubated at 4°C for 40 min. The slides were then placed randomly side by side in a horizontal electrophoresis chamber (Fisher Scientific, Fisher Biotech Recirculating Large Horizontal System #FB-SBR-2025). The chamber was filled with cold (4°C) electrophoresis solution, and electrophoresis proceeded for 40 min at 25 V and 300 mA (Electrophoresis Power Supply, EPS 600; Pharmacia Biotech, Piscataway, NJ, USA). Up to this point, all steps of the comet assay were performed under dim light to minimize artificial induction of DNA single strand breaks.

Following electrophoresis, the slides were rinsed four times with neutralization solution (0.4 M Tris, pH 7.5). If necessary, the slides were stored in neutralization solution in sealed containers at 4°C for up to 1 week. After draining the neutralization solution from the slides, 100 μ l of 4 μ g/ml ethidium bromide (Sigma) was pipetted onto each slide to stain the DNA, and a new coverslip was applied. After 5 min, the coverslips were removed, each slide was rinsed with a small amount of deionized distilled water, 2 additional drops of 4 μ g/ml ethidium bromide were pipetted onto each slide, and new coverslips applied. If necessary, the slides were stored in air-tight containers for up to 3 days before analysis.

The ethidium bromide-stained DNA was visualized using a fluorescence microscope equipped with a 515–560 nm excitation filter and a 590 nm barrier filter, connected through a gated CCD camera to a computer-aided image analyzer. Komet 3.1 imaging software (Kinetic Imaging Ltd., Liverpool, UK) was used for image analysis. Results were expressed as the comet moment (product of comet tail length and intensity of the tail).

Data Analysis

Data are summarized as mean \pm SEM of comet moment from 5 separate experiments that were conducted independently. In each experiment and for each PBL sample, 2 slides were prepared, and 25 cells/slide were selected at random for analysis using the comet assay.

Statistical analyses were conducted using the SigmaStat, version 2.0 computer program (Jandel Scientific Corporation, San Rafael, CA, USA). First, a three-way analysis of variance (ANOVA) was used on all the data to determine whether there were significant differences in comet moment a) between treatments (cells exposed to SM alone, H₂O₂ alone, or SM plus H₂O₂), b) between time points following SM exposure, and c) between cells exposed to different concentrations of SM. Significant two-way interactions were observed, and a two-way ANOVA was therefore performed on each treatment group, comparing the comet moment at different times following SM exposure and the comet moment of cells exposed to different SM concentrations. Significant two-way interactions in each treatment group were observed, and a one-way ANOVA was subsequently used to assess differences in comet moment between SM concentrations at each time point for each treatment group. A one-way ANOVA was again used to determine whether there were differences in comet moment between different time points following SM exposure at each SM concentration for each treatment group. A one-way ANOVA was also used to assess differences in comet moment between treatments at each SM concentration and each time point. When a significant difference was found, Newman-Keuls multiple comparison method was used to identify pairwise differences. Differences were considered significant at $p < 0.05$.

For viability determinations, each PBL sample was assayed in triplicate.

RESULTS

DNA Damage Detected in PBL Exposed to SM Alone

PBL exposed to SM demonstrated a SM concentration- and time-dependent increase in detectable DNA damage (Fig. 1). Compared with control cells, a significant increase in DNA damage was detected at SM concentrations ≥ 600 μ M at 2 h, ≥ 300 μ M at 4 h, and ≥ 60 μ M at 6 h.

The amount of DNA single strand breaks observed in control cells was minimal (Figs. 1 and 3) and did not significantly change over the 6 h of incubation. Although not statistically significant, a consistent pattern was observed throughout all of the 2 and 4 h experiments in which DNA single strand breakage measured in control cells was higher than that measured in cells exposed to SM concentrations ≤ 100 μ M. At 6 h, DNA single strand breakage detected at ≤ 100 μ M SM was equivalent to that of control cells.

Detectable DNA damage did not differ significantly between the 2- and 4-h time points in cells exposed to ≤ 100 μ M SM; at 6 h, detectable DNA damage was significantly higher than that measured at 2 or 4 h after exposure to ≥ 60 μ M SM. However, there was no significant difference between the 4- and 6-h time points in strand breakage detected following exposure to 600 or 1000 μ M SM.

Over 6 h following SM exposure, viability remained above 90%.

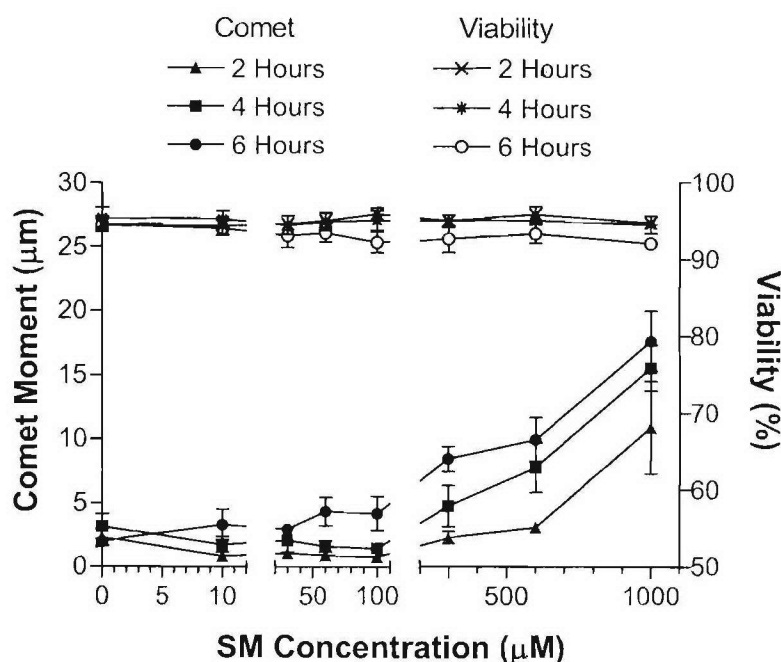


FIG. 1. Effect of sulfur mustard (SM) concentration on detectable DNA damage and on viability over time following SM exposure. Peripheral blood lymphocytes (PBL) were exposed to buffer (control) or to buffer plus the indicated SM concentrations, harvested at 2, 4, or 6 h after exposure, and the comet assay then performed. For each PBL sample, slides were prepared in duplicate, and 25 cells/slide were analyzed. Data are mean \pm SEM of comet moment of 5 separate experiments (250 comets analyzed for each data point). There was a SM concentration- and time-dependent increase in the detectable amount of DNA single strand breakage in PBL exposed to ≥ 600 μ M SM at 2 h, ≥ 300 μ M SM at 4 h, and ≥ 60 μ M SM at 6 h. Viability was $>90\%$ at all SM concentrations and time points tested.

DNA Damage Detected in PBL Exposed to H₂O₂ Alone

DNA damage in H₂O₂-exposed cells was approximately 11 times larger than that in control cells (Fig. 2A–C; 0 μ M SM). There was no significant difference in DNA damage measured in cells exposed to H₂O₂ at 2, 4, or 6 h after PBL isolation (Fig. 2A–C).

The amount of DNA single strand breaks observed in cells exposed to H₂O₂ alone was significantly larger than that observed in SM- or SM/H₂O₂-exposed cells at all time points and all SM concentrations tested (Fig. 2A–C). At 4 and 6 h after SM exposure, detectable DNA damage averaged 1.7 times larger for cells exposed to H₂O₂ alone than for cells exposed to 1000 μ M SM, with or without H₂O₂; it was 2.6 times larger at 2 h after exposure.

Viability of cells exposed to H₂O₂ alone at 2, 4, or 6 h after PBL isolation remained above 90% and averaged 94% at each time point.

DNA Damage Detected in PBL Exposed to SM Followed by H₂O₂

As SM concentration increased, detectable DNA damage in H₂O₂-exposed cells decreased up to 30 μ M SM at each time point (Fig. 2). At SM concentrations ≥ 30 μ M, strand breakage measured in cells exposed to both SM and H₂O₂ was not significantly different from that in cells exposed to SM alone. The amount of measurable DNA damage in cells exposed to H₂O₂ increased over time following SM exposure and with increasing SM concentrations ≥ 300 μ M.

In photomicrographs, control cells appeared to show the least amount of DNA damage, followed by cells exposed to SM alone and cells exposed to both H₂O₂ and SM (Fig. 3). The most damage appeared in those cells exposed to H₂O₂ alone.

The viability of cells exposed to both SM and H₂O₂ remained above 85% at all time points and SM concentrations tested.

DISCUSSION

This study shows that, in the comet assay, SM interferes with DNA migration and detection of DNA single strand breaks. This is presumably due to the formation of SM-induced DNA cross-links.

It appears that DNA cross-linking is SM concentration-dependent up to 30 μ M, with maximum DNA cross-linking occurring with an exposure between 10 and 30 μ M SM. This is suggested by the fact that, up to 30 μ M SM, there is a SM concentration-dependent decrease in the detection of H₂O₂-induced DNA single strand breaks at all time points tested. Similar results were obtained in an earlier study in which cross-linking was detected in the DNA of SM-exposed rat cutaneous epidermal keratinocytes using an alkaline DNA unwinding assay (Sorscher and Conolly 1989). These investigators found that DNA cross-linking did not increase at SM concentrations higher than 5 μ M. The lowest SM concentration at which maximum DNA cross-linking occurs may depend upon cell type.

At SM concentrations ≥ 30 μ M, detection of DNA single strand breaks produced by SM plus H₂O₂ is no different from that

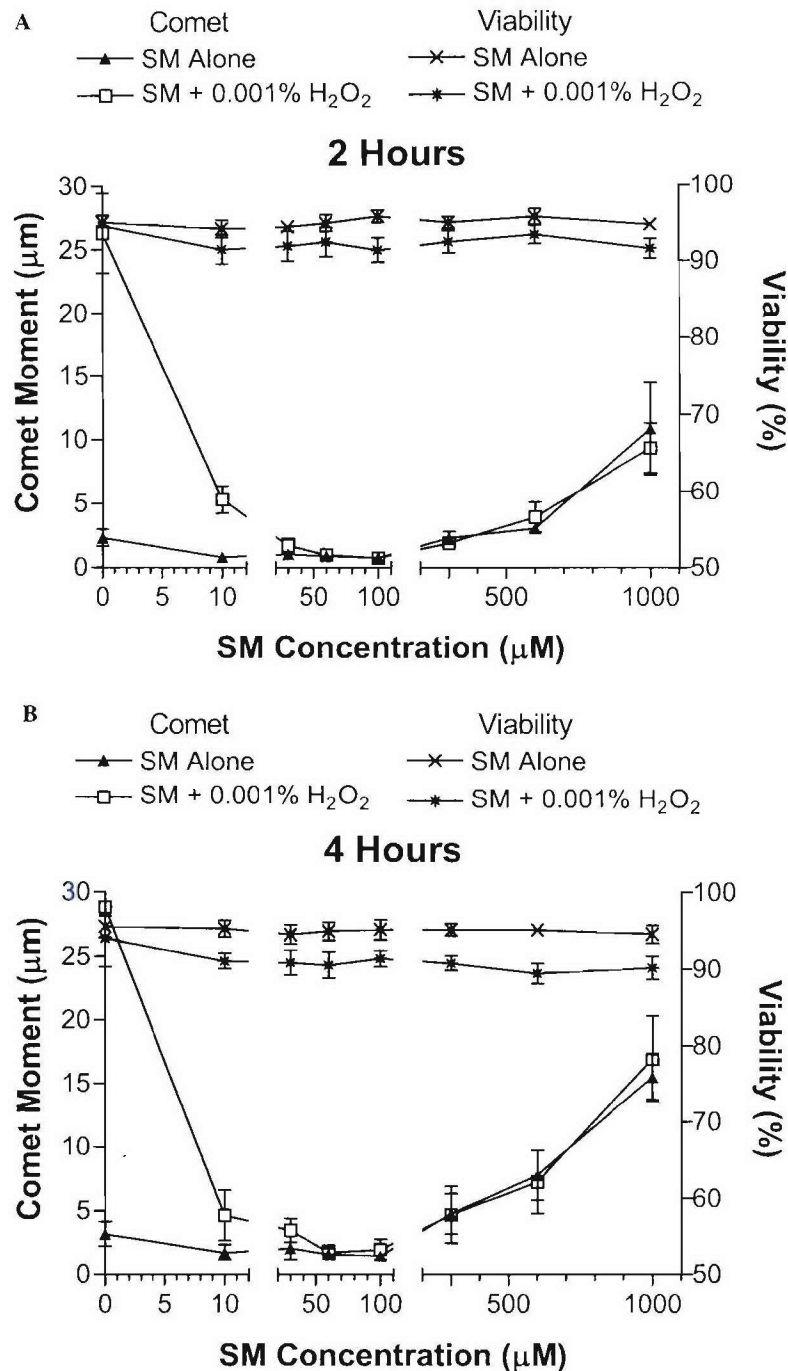


FIG. 2. Effect of sulfur mustard (SM) concentration on detectable DNA damage and on viability in H₂O₂-exposed cells. Peripheral blood lymphocytes (PBL) were exposed to either buffer (control); buffer plus a 5-min exposure to 0.001% H₂O₂ at 2, 4, or 6 h after PBL isolation; buffer plus the indicated SM concentrations; or buffer plus the indicated SM concentrations followed by a 5-min exposure to 0.001% H₂O₂ at 2, 4, or 6 h after SM exposure. PBL exposed to SM alone were harvested at 2, 4, or 6 h after exposure. PBL exposed to H₂O₂ alone or SM plus H₂O₂ were harvested immediately after exposure to H₂O₂. Cells were analyzed with the comet assay. For each PBL sample, slides were prepared in duplicate, and 25 cells/slide were analyzed. Data are mean \pm SEM of comet moment of 5 separate experiments (250 comets analyzed for each data point). At all time points tested, the amount of DNA damage measured was significantly larger in cells exposed to H₂O₂ alone than in SM- or SM/H₂O₂-exposed cells. In cells exposed to H₂O₂ plus SM, there was a SM concentration-dependent decrease in the detectable amount of single strand breaks up to 30 μ M SM. At 30 μ M SM and above, the amount of DNA damage measured in cells exposed to H₂O₂ plus SM was similar to that in cells exposed to SM alone. Viability of cells exposed to H₂O₂ alone was $>90\%$ at each time point. For cells exposed to H₂O₂ plus SM, viability was $>85\%$ at each time point and SM concentration tested. (Continued)

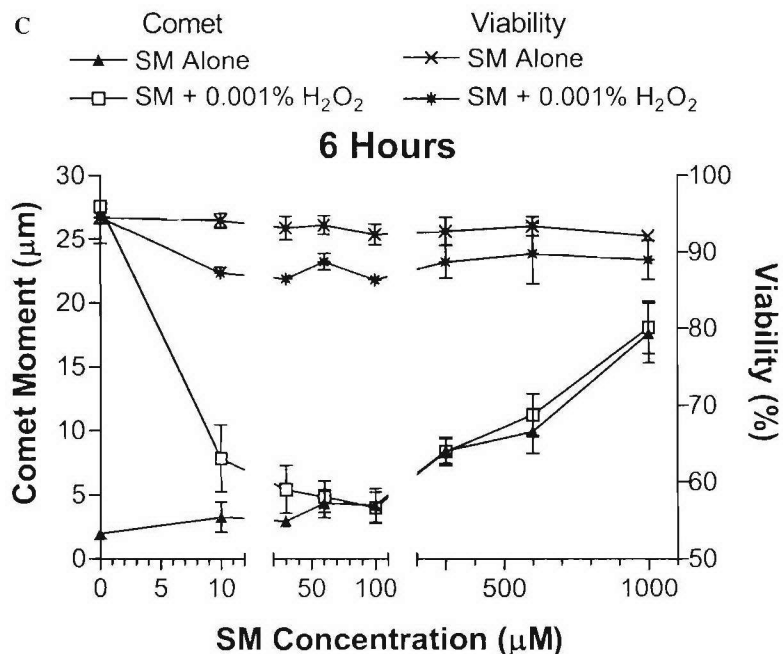


FIG. 2. Continued.

produced by SM alone. This indicates that detection of H_2O_2 -damaged DNA is completely masked by SM-induced cross-links and that the concentration-dependent increase in detectable DNA damage observed at SM concentrations $\geq 100 \mu\text{M}$ SM reflects only the damage produced by SM. At high concentrations of SM, the amount of DNA single strand breakage is apparently so great that the effect of cross-linking does not completely prevent detection of strand breaks. Therefore, although DNA damage produced by $1000 \mu\text{M}$ SM appears lower than that produced by 0.001% H_2O_2 alone, the amount of DNA damage produced by $1000 \mu\text{M}$ SM may actually be close to the same or higher than that produced by 0.001% H_2O_2 , the true amount of damage being concealed by the presence of SM-induced cross-links.

The migration of DNA from cells exposed to lower concentrations of SM in the absence of H_2O_2 appears to be completely inhibited by DNA cross-linking. Compared with control PBL in which a very small amount of DNA single-strand breakage is expected (Jostes et al. 1989) and which is, in fact, detected with the comet assay, the DNA of cells exposed to lower concentrations of SM consistently demonstrates either less than or the same amount of DNA damage as control cells.

The time-dependent increase in detection of DNA single strand breaks following SM exposure may be due to an increase in the actual number of DNA single strand breaks produced by SM and/or an increase in the detectable number of DNA single strand breaks as a result of DNA cross-link repair. It is assumed that repair of both DNA single strand breaks and DNA cross-links occurs over time, which would either decrease or increase, respectively, the amount of DNA damage reflected in the comet assay. Since there is an overall increase in the amount of DNA damage detected over time, cross-link repair appears to take

place sooner and/or at a higher rate than strand break repair. Previous studies with rat cutaneous keratinocytes suggest that at low SM concentrations, cells are capable of completely repairing SM-induced DNA damage within 22 h following exposure, but at high concentrations the damage is so extensive that the cell loses its ability to completely repair damaged DNA (Ribeiro et al. 1991). Up to at least 6 h following SM exposure, the rate of DNA single strand break formation may be higher than the rate of DNA single strand break repair, which would also contribute to the overall effect.

At 6 h following SM exposure, detection of H_2O_2 -induced DNA damage is prevented to a large extent by SM. This indicates that a significant number of DNA cross-links are still present at 6 h after SM exposure. Indeed, in studies using SM-treated human epidermal keratinocytes (Mol et al. 1993) and various types of mammalian cells treated with nitrogen mustard (Ewig and Kohn 1977; Ross et al. 1978; Murnane and Byfield 1981), it was observed that most SM- or nitrogen mustard-induced cross-links are repaired within 24 h after exposure; however, some cross-links are persistent and remain at 24 h, independent of the concentration of the agent. Evidence indicates that the ability to repair DNA cross-links may depend upon the phase in the cell cycle during which cells are exposed (Lin et al. 1996).

SM and H_2O_2 concentrations employed in these experiments are not cytotoxic within the times to which the PBL were exposed. As previously reported (Henderson et al. 1998), to avoid false positive responses, the viability of cells used in the comet assay should exceed 75% since cytotoxins induce an increase in DNA migration when cell viability is $\leq 75\%$.

In the comet assay, SM-induced DNA cross-linking inhibits the migration of H_2O_2 -damaged DNA at concentrations of SM

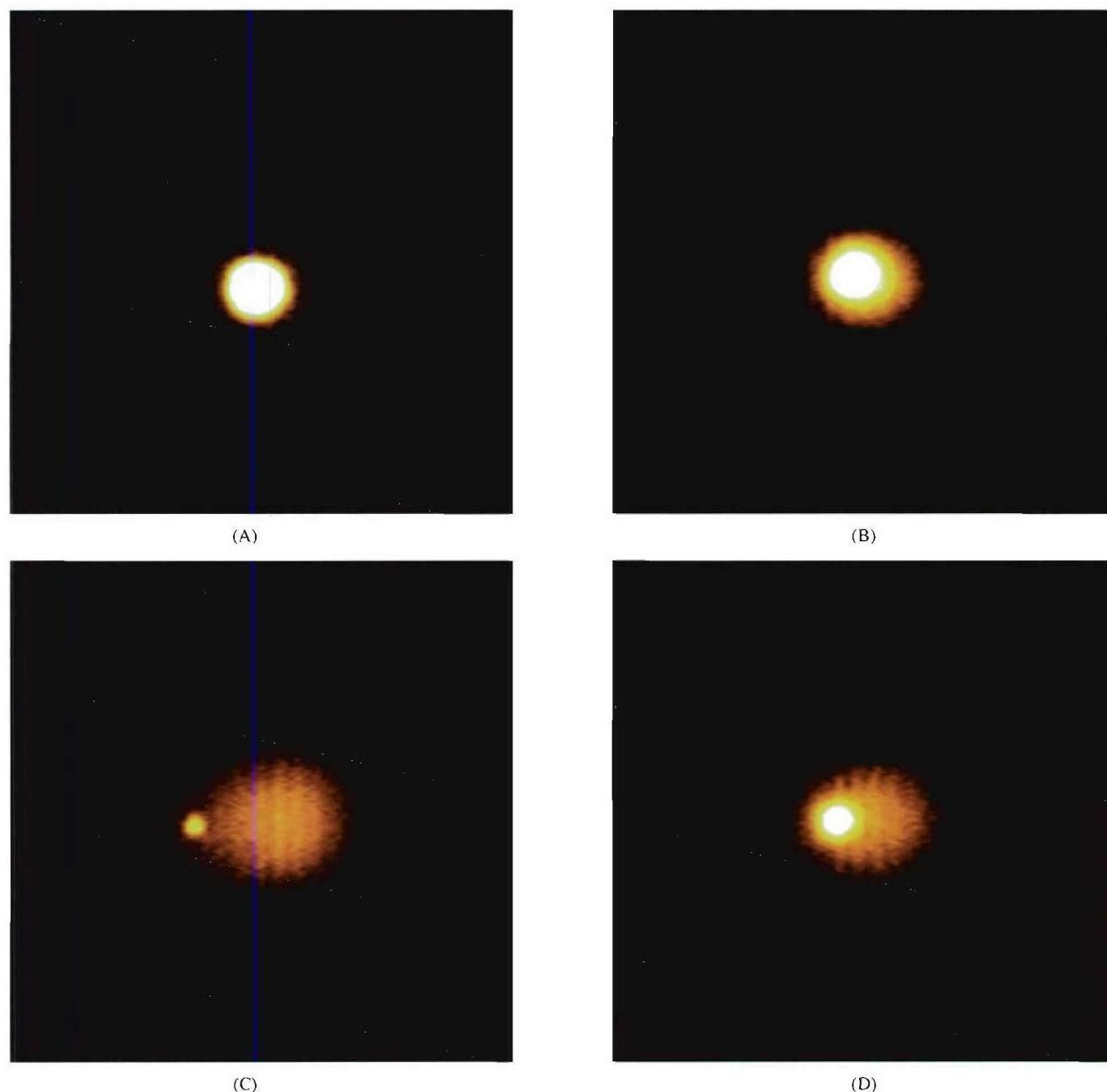


FIG. 3. Photomicrographs of comets from cells exposed to sulfur mustard (SM) and/or H_2O_2 . Peripheral blood lymphocytes (PBL) were exposed to A) buffer (control), B) buffer plus 1000 μM SM, C) buffer plus a 5-min exposure to 0.001% H_2O_2 at 2 h after PBL isolation or D) buffer plus 1000 μM SM followed 2 h later by a 5-min exposure to 0.001% H_2O_2 . Cells exposed to SM alone were harvested at 2 h after exposure. Cells exposed to H_2O_2 alone or SM plus H_2O_2 were harvested immediately after H_2O_2 exposure. The comet assay was then performed. For visualization of DNA damage, observations were made of ethidium bromide-stained DNA using a fluorescent microscope equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. Cells are magnified 1000 \times . Length and fluorescence intensity of the comet tail denote the relative amount of DNA single strand breaks (longer length and higher intensity indicate more DNA damage). The anode is to the right. There appeared to be less DNA damage in cells exposed to H_2O_2 plus SM (D) than in those exposed to H_2O_2 alone (C). Apart from the control (A), cells exposed to SM alone (B) showed the least amount of DNA damage.

at which there is no detectable DNA damage due to SM alone. This implies that even when no DNA migration is apparent in the comet assay, there is still significant SM-induced damage. This is in agreement with the findings of others who evaluated by electrophoresis total genomic DNA extracted from SM-exposed PBL (Meier and Millard 1998); DNA fragmentation

was detected as early as 2 h after exposure to 300 μM SM. In another study, using the nucleoid sedimentation assay, it was demonstrated that within 1 h of exposure to as little as 0.1 μM SM, the structural integrity of DNA from rat cutaneous keratinocytes was compromised, assumed to be the result of DNA single-strand breakage (Ribeiro et al. 1991).

Due to DNA cross-linking, the comet assay as performed in this study does not reflect the full extent of DNA damage induced by SM and is therefore limited in its ability to detect DNA single strand breaks in SM-exposed cells. To directly assess the relative amount of DNA single strand breaks in SM-exposed cells, assay modification to eliminate cross-links or to correct for the presence of cross-links is indicated.

The ability of SM to impede the migration of H₂O₂-damaged DNA is an indication of the amount of SM-induced DNA cross-linking. Thus, with the utilization of H₂O₂ or other compounds that create DNA single-stranded breaks, the comet assay may potentially be used to provide information about the induction of DNA cross-links and their repair in cells exposed to SM.

REFERENCES

- Clayson, E. T., Kelly, S. A., and Meier, H. L. 1993. Effects of specific inhibitors of cellular functions on sulfur mustard-induced cell death. *Cell Biol. Toxicol.* 9:165-175.
- de With, A., Leitz, G., and Greulich, K. O. 1994. UV-B-laser-induced DNA damage in lymphocytes observed by single-cell gel electrophoresis. *J. Photochem. Photobiol. B: Biol.* 24:47-53.
- Ewig, R. A. G., and Kohn, K. W. 1977. DNA damage and repair in mouse leukemia L1210 cells treated with nitrogen mustard, 1,3-bis-(2-chloroethyl)-1-nitrosourea, and other nitrosoureas. *Cancer Res.* 37:2114-2122.
- Fairbairn, D. W., Olive, P. L., and O'Neill, K. L. 1995. The comet assay: A comprehensive review. *Mutat. Res.* 339:37-59.
- Freeman, S. E., Blackett, A. D., Monteleone, D. C., Setlow, R. B., Sutherland, B. M., and Sutherland, J. C. 1986. Quantitation of radiation-, chemical-, or enzyme-induced single strand breaks in nonradioactive DNA by alkaline gel electrophoresis: Application to pyrimidine dimers. *Anal. Biochem.* 158:119-129.
- Gedik, C., Ewen, S., and Collins, A. 1992. Single-cell gel electrophoresis applied to the analysis of UV-C damage and its repair in human cells. *Int. J. Radiat. Biol.* 62:313-320.
- Henderson, L., Wolfreys, A., Fedyk, J., Bournier, C., and Windebank, S. 1998. The ability of the comet assay to discriminate between genotoxins and cytotoxins. *Mutagenesis* 13:89-94.
- Jostes, R., Reese, J. A., Cleaver, J. E., Molero, M., and Morgan, W. F. 1989. Quiescent human lymphocytes do not contain DNA strand breaks detectable by alkaline elution. *Exp. Cell Res.* 182:513-520.
- Kent, C. H. R., Eady, J. J., Ross, G. M., and Steel, G. G. 1995. The comet moment as a measure of DNA damage in the comet assay. *Int. J. Radiat. Biol.* 67:655-660.
- Lin, P., Vaughan, F. L., and Bernstein, I. A. 1996. Formation of interstrand DNA cross-links by bis-(2-chloroethyl)sulfide (BCES): A possible cytotoxic mechanism in rat keratinocytes. *Biochem. Biophys. Res. Commun.* 218:556-561.
- Meier, H. L., Gross, C. L., and Papirmeister B. 1987. 2,2'-dichlorodiethyl sulfide (sulfur mustard) decreases NAD⁺ levels in human leukocytes. *Toxicol. Lett.* 39:109-122.
- Meier, H. L., and Johnson, J. B. 1992. The determination and prevention of cytotoxic effects induced in human lymphocytes by the alkylating agent 2,2'-dichlorodiethyl sulfide (sulfur mustard, HD). *Toxicol. Appl. Pharmacol.* 113:234-239.
- Meier, H. L., and Millard, C. B. 1998. Alterations in human lymphocyte DNA caused by sulfur mustard can be mitigated by selective inhibitors of poly(ADP-ribose) polymerase. *Biochim. Biophys. Acta* 1404:367-376.
- Mol, M. A. E., van der Schans, G. P., and Lohman, P. H. M. 1993. Quantification of sulfur mustard-induced DNA interstrand cross-links and single-strand breaks in cultured human epidermal keratinocytes. *Mutat. Res.* 294:235-245.
- Murnane, J. P., and Byfield, J. E. 1981. Irreparable DNA cross-links and mammalian cell lethality with bifunctional alkylating agents. *Chem. Biol. Interact.* 38:75-86.
- Olive, P. L., Wlodek, D., Durand, R. E., and Banath, J. P. 1992. Factors influencing DNA migration from individual cells subjected to gel electrophoresis. *Exp. Cell Res.* 198:259-267.
- Olive, P. L., and Banath, J. P. 1993. Induction and rejoining of radiation induced DNA single strand breaks: Tail moment as a function of position in the cell cycle. *Mutat. Res. (DNA repair)* 294:275-283.
- Olive, P. L., and Banath, J. P. 1995. Sizing highly fragmented DNA in individual apoptotic cells using the comet assay and a DNA crosslinking agent. *Exp. Cell Res.* 221:19-26.
- Papirmeister, B., Feister, A. J., Robinson, S. I., and Ford, R. D. 1991. Chemistry of sulfur mustard. In *Medical Defense Against Mustard Gas* (Editors), 91-122. Boston: CRC Press.
- Pfuhler, S., and Wolf, H. U. 1996. Detection of DNA-crosslinking agents with the alkaline comet assay. *Environ. Mol. Mutagen.* 27:196-201.
- Reid, B. D., and Walker, I. G. 1969. The response of mammalian cells to alkylating agents II. On the mechanism of the removal of sulfur-mustard-induced crosslinks. *Biochim. Biophys. Acta* 179:179-188.
- Ribeiro, P. L., Mitra, R. S., and Bernstein, I. A. 1991. Assessment of the role of DNA damage and repair in the survival of primary cultures of rat cutaneous keratinocytes exposed to bis(2-chloroethyl)sulfide. *Toxicol. Appl. Pharmacol.* 111:342-351.
- Ross, W. E., Ewig, R. A. G., and Kohn, K. W. 1978. Differences between melphalan and nitrogen mustard in the formation and removal of DNA cross-links. *Cancer Res.* 38:1502-1506.
- Singh, N. P., McCoy, M. T., Tice, R. R., and Schneider, E. L. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* 175:184-191.
- Slamenová, D., Gäbelová, A., Ruzeková, L., Chalupa, I., Horváthová, E., Farkasová, T., Bozsakyová, E., and Stetina, R. 1997. Detection of MNNG-induced DNA lesions in mammalian cells; validation of comet assay against DNA unwinding technique, alkaline elution of DNA and chromosomal aberrations. *Mutat. Res.* 383:243-252.
- Smith, W. J., Sanders, K. M., Gales, Y. A., and Gross, C. L. 1991. Flow cytometric analysis of toxicity by vesicating agents in human cells *in vitro*. *J. Toxicol. Cutan. Ocul. Toxicol.* 10:33-42.
- Sorscher, D. H., and Conolly, R. B. 1989. Pretreatment of primary rat cutaneous epidermal keratinocyte culture with a low concentration of MNNG: Effect on DNA cross-linking measured *in situ* after challenge with bis-2-chloroethyl sulfide. *J. Toxicol. Environ. Health.* 27:367-379.
- Szmigiero, L., and Studszian, K. 1988. H₂O₂ as a DNA fragmenting agent in the alkaline elution interstrand crosslinking and DNA-protein crosslinking assays. *Anal. Biochem.* 168:88-93.
- Walker, I. G. 1971. Intrastrand bifunctional alkylation of DNA in mammalian cells treated with mustard gas. *Can. J. Biochem.* 49:332-336.
- Walker, I. G., and Reid, B. D. 1971. Some properties of sublines of L-cells with a decreased sensitivity to bis(2-chloroethyl)sulfide. *Cancer Res.* 31:510-515.